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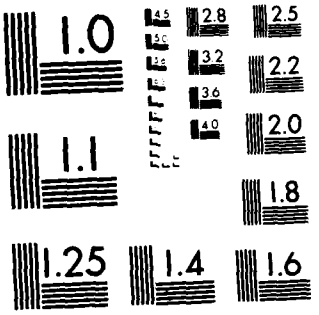

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ANNUAL REPORT

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NEW YORK BLOOD CENTER
NEW YORK, NY

Enzymatic Conversion of Red Cells for Transfusion

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Human In vivo Studies with Alapha-Galactosidase Converted Erythrocytes.

We have previously reported the results of our initial pre-clinical investigation with enzymatically converted human type B erythrocytes. That study consisted of three volunteers, a type B individual whose cells were labeled with Cr^{51}, enzymatically converted to type O and 1 ml aliquots returned to him as well as administered to type O and A recipients. The primary objective was to determine the longevity in vivo of such enzyme treated cells. Having shown that they do survive normally under such conditions, we have begun studies to explore their possible immunogenicity. Our second preclinical study just recently completed (in collaboration with Drs. Reich and Mayer of Memorial Hospital Sloan Kettering) again consisted of three normal human volunteers representing each blood group. Two mls of converted B cells were transfused initially to the A and O recipients and the B donor. Two weeks later another two mls were given and then a final one ml of enzymatically converted and Cr^{51} labeled red cells was administered to each participant two weeks after the second dose. Thus a total of five mls of converted cells was transfused over a period of four weeks. Serological testing was performed at intervals prior to, during and for seven weeks after the three transfusions. These in vitro assays revealed no increase in anti B IgM and IgG iso-haemagglutinin titers following transfusions. Also, their post transfusion sera did not agglutinate or lyse treated cells. Similarly, no antibodies were detected against coffee bean alpha-galactosidase as determined by an immunodiffusion assay and a very sensitive enzyme-linked immunoassay (ELISA) procedure. The latter can detect anti alpha-galactosidase antibody at a 1:2000 dilution of serum prepared from immunized rabbits when tested against 5 micrograms of enzyme as antigen. The procedure basically consists of coupling the antigen to well-surfaces of specially prepared plastic plates, blocking any remaining free sites in the wells with bovine serum albumin and then reacting the bound antigen with appropriately titered human or rabbit serum containing antibody. After washing to remove excess antibody the plates are incubated with peroxidase conjugated goat IgG antibody specific for either human or rabbit IgG, washed again and the bound peroxidase in the wells allowed to react with a substrate (O-Dianisidine) which produces a color reaction that can be measured quantitatively in a specially modified spectrophotometer. As evidence of lack of a detectable in vivo immuno response there were no significant differences in the survival of labeled converted cells in the B donor and the A or O recipients; the 24 hour percent survival ranging from 86% for the O recipient to 88% for both the B donor and the A recipient and the fifty percent survival times ranging from 26.5 days for both recipients to 29.5 days for the B donor receiving his own cells. Furthermore, the shapes of the Cr^{51} survival curves were similar for all three participants and did not show any downward shifts in slope which would have indicated an increased rate of cell destruction due to nascent antibody formation. It would thus appear that treated cells are not immunogenic under these conditions.

This aspect of our work is being transitioned to 6.3 funding so that further preclinical and clinical studies with larger amounts of converted cells will be performed under the aegis of NMRDC.

Alpha-N-Acetylgalactosaminidase and Conversion of Group A Cells.

We reported last year our finding an alpha-N-acetylgalactosaminidase (A-zyme) in pigeon and chicken livers which removes A antigenicity from type A

cells. We have now developed a relatively straightforward procedure to prepare the quantities of enzyme needed for our current studies. Fresh chicken livers are trimmed of extraneous fat and dehydrated either by extraction with acetone or by freeze drying. The dried and powdered chicken liver (140 gm) is mixed with 0.01 M sodium acetate pH 4.0 at 4° C and blenderized (10-30') in order to insure a fine suspension which is allowed to settle (2-16 hr). Recovery of the supernatant is effected by centrifugation. Solid ammonium sulfate is then slowly added to the supernatant to a concentration of 30%. the resulting precipitate is centrifuged away and ammonium sulfate is again added to a final concentration of 50% yielding a precipitate containing as part of a mixture of other enzymes and proteins our enzyme of interest, alpha-N-acetylgalactosaminidase. The precipitate is pelleted by centrifugation, dialyzed against 0.01 M sodium acetate pH 5.0 to remove the remaining ammonium sulfate and allow the pellet to dissolve. Contaminating substances are then separated from the alpha-N-acetylgalactosaminidase by subjecting the solubilized mixture first to ion exchange chromatography and then gel filtration. Specifically, a volume of the mixture containing 1500-1800 units of alpha-N-acetylgalactosaminidase is applied to a column (5 x 16 cm, 300 ml) of the cation exchanger, carboxymethylcellulose (CM-52 Whatman) equilibrated in 0.01 M sodium acetate pH 5.0. Following washing of the column with 0.05 M sodium acetate ph 5.0, a linear gradient ranging from 0.07 M sodium acetate to 0.20 M sodium acetate pH 5.0 is applied (750 ml of each) which results in the elution of a protein fraction containing alpha-N-acetylgalactosaminidase. This fraction is then dialyzed against 0.01 M potassium phosphate buffer pH 6.0 and applied to a column (2.5 x 13 cm, 60 ml) containing the anion exchanger diethylaminoethyl Sephadex (A-50 Pharmacia) equilibrated with the same buffer. The column is then washed with this buffer resulting in the elution of an alpha-N-acetylgalactosaminidase containing-fraction which is concentrated and subjected to gel filtration through a column (2.5 x 10 cm) containing porous agarose (Sephadex G100, Pharmacia). The elution region containing the enzyme is concentrated and subjected once more to gel filtration, this time using a porous polyacrylamide-agarose mixture containing column (BioGel P-150, Bio Rad; 2.5 x 150). The enzyme fraction thus obtained is stable and has been purified free of any detectable contaminating proteases and exoglycosidases including sialidase. The purified enzyme does exhibit alpha-galactosaminidase activity (8-10%) which we believe to be intrinsic and not due to a contaminating B-zyme. Similar results and conclusions have been reported by other workers studying various isolated mammalian A-zymes (Kusiak, Quirk,Brady 1978; Dean, Sweely 1979).

We have shown that chicken liver A-zyme aside from hydrolyzing synthetic substrate (p-nitrophenylglycoside of N-acetylgalactosamine) also is effective with group A containing salivary glycoproteins; the Forssman antigen which is a glycolipid pentasaccharide containing a terminally linked alpha-N-acetylgalactosamine residue, and blood group A glycolipids isolated from dog and human intestines.

It can also remove A antigenicity from the surface of group A erythrocytes. There are three major A subtypes: A_1, A_{int}, A_2. They are believed to differ in the number of antigenic sites, A_1 cells having the most, and possibly in the kind of antigenic structures, A_1 cells having some which may not be present in the other two. We were able to show that group A_2, A_{int}

and A_2B erythrocytes were converted to either type O or type B cells as measured by lack of agglutination with anti-A antiserum. We found, however, that A_1 cells treated under the same conditions did not lose all of their antigenicity since they remained agglutinable with anti-A antiserum up to a five-fold dilution. The remaining antigens could be removed but only under stringent conditions which rendered the converted A_1 cells unfit for transfusion.

We are currently pursuing the development of proper conversion conditions for A_1 cells so that we can proceed to similar studies of converted A cells as reported here for converted B cells. Our approaches include: varying pH, temperature and buffer composition. Treatment with A-zyme plus other enzymes such as endo-beta-galactosidase, since such combinations might either remove A antigenicity unreactive to A-zyme or modify those antigenic structures possibly inhibiting the action of A-zyme. An endo-beta-galactosidase from Escherichia freundi has been shown to reduce A, B, H (Fukuda, Hakamori 1979) as well as I and i antigenicity (Dôinel et al. 1980) on the surface of red cells. And finally, since there are reports in the literature (Li,Hirabayashi, Li, 1981; Paschke, Kress, 1982) of a protein activator which can, particularly with glycolipid substrates, increase the activity of some exoglycosidases from mammalian livers, we are attempting to isolate such an activator from chicken liver in order to determine whether it can enhance the effect of A-zyme upon A_1 cells. Thus far the first two approaches have been most actively studied and have yielded encouraging results. Our original buffer system developed for alpha-galactosidase conversion of B cells, contains citrate, phosphate and 0.15 M sodium chloride. This was the buffer we used for our initial enzymatic conversions of A cells. Recent hemagglutination inhibition studies with blood type A active glycoprotein indicated, however, that the presence of saline in this buffer significantly reduced the rate of removal of A antigenicity from the glycoprotein. We now have preliminary evidence that by using buffer systems devoid of sodium chloride A_1 cells can be converted to type O with the chicken liver A-zyme. We have also begun studies with the endo-beta-galactosidase mentioned earlier. We find that while it, when used alone, removes very little A antigenicity from A_1 cells it can, in combination with chicken liver A-zyme under certain conditions. reduce the time needed to convert A_1 cells to O as compared to when A-zyme is used alone. Further studies are being carried out in order to standardize conditions for enzymatic conversion of A_1 cells so that in vivo metabolic and membrane studies can be performed in an analogous manner to those done with converted type B erythrocytes.

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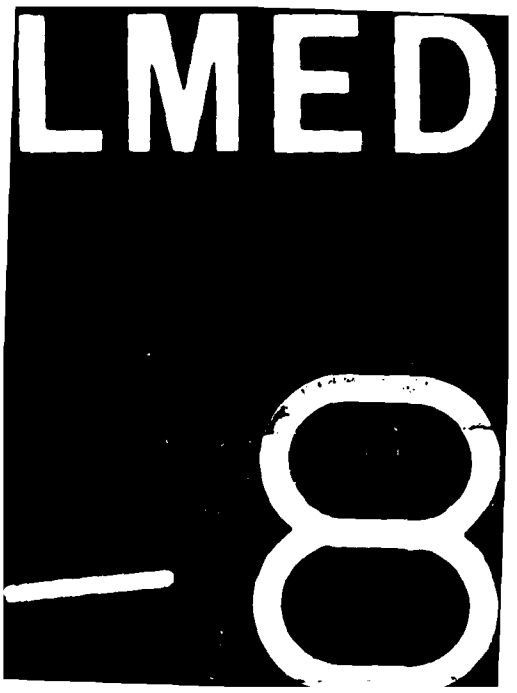
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